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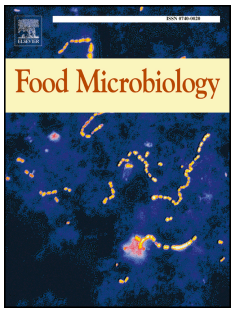

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PII: S0740-0020(18)30844-X

DOI: https://doi.org/10.1016/j.fm.2019.03.021

Reference: YFMIC 3196

To appear in: *Food Microbiology*

Received Date: 5 September 2018

Revised Date: 15 March 2019

Accepted Date: 18 March 2019

Please cite this article as: Schmidt, M., Arendt, E.K., Thery, T.L.C., Isolation and characterisation of the antifungal activity of the cowpea defensin Cp-thionin II, *Food Microbiology* (2019), doi: https://doi.org/10.1016/j.fm.2019.03.021.

Isolation and characterisation of the antifungal activity of the cowpea defensin Cp-thionin II

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ABSTRACT

As a result of the rapidly growing human population, reducing post-harvest crop losses of cereals due to microbial pests has major importance. Plant defensins have the potential to fulfil these demands, being highly specific and efficient antimicrobial agents. Hence, this study aimed to extract and characterise a peptide from cowpea seeds and investigate its antifungal performance. After extraction and partial purification, N-terminal sequencing was used to identify the primary peptide in the extract as cowpea-thionin II. Antifungal activity *in vitro* was found against *Fusarium culmorum* (MIC = 50 µg/mL), but *Aspergillus niger* and *Penecillium expansum* showed an MIC > 500 µg/mL. The extract was resistant against heat treatment (100℃, 15 min) but lost its antifungal activity in presence of cations (Na^+, K^+, Ca^{2+} and Mg^{2+}, respectively). Membrane permeabilization of fungal hyphae was evident at 25 µg/mL, while induction of oxidative stress only had minor contribution to the antifungal performance. The extract did not induce haemolysis at all concentrations tested (up to 200 µg/mL). Finally, it was successfully used to protect stored wheat grains from fungal spoilage (determined via ergosterol content) when applied at 100 µg/mL. In conclusion, the defensin Cp-thionin II showed the potential for future application as food bio-preservative.

1. INTRODUCTION

Satisfying the nutritional demands of the rapidly growing global population has, during recent decades, turned into an ever increasing challenge. Due to the limited resources and agricultural area available, research has focused on improved efficiency in terms of food production and preservation. It was estimated that with the current amounts of food waste, the food production has to increase by 60 - 110% until 2050, in order to feed the population worldwide (Ray et al., 2013; Tilman et al., 2011). The reason behind this rapid increase is not only the growing population, but also the predicted increase in food consumption per capita (Tilman & Clark, 2014). In addition, recent studies have demonstrated that the approaches to increase the yield of agricultural crops, such as cereals, are not sufficient and sustainable to satisfy the global demands of future generations (Ray et al., 2013). Considering the importance of cereals for human nutrition over the last centuries (approximately 70 kg per person per year), this poses a major problem (Albertson et al., 2015). Hence, research and industry are trying to reduce crop losses and increase the sustainability.

One of the main reasons of food waste is the microbial spoilage of the crops in-field, as well as post-harvest. According to Freita-Silva et al. (2014), approximately 15% of the global cereal production is lost due to microbial spoilage. While the reduction of in-field spoilage is intensely investigated (Mannaa & Kim, 2017), the equally important microbial protection after harvest and during storage is often overlooked. However, up to 20% of the harvested cereals worldwide turn into food waste, mainly as a result of microbial spoilage during storage and downstream processing (Ridolfi et al., 2018). Microbial contaminants include bacteria, yeasts and filamentous fungi. For cereals, fungi belonging to the genera of *Fusarium*, *Aspergillus* and *Penicillium* are the most commonly found spoilage organisms (Russo et al., 2017). Growth and development of these fungi during storage result in grain quality deterioration and, if not disposed, potential consumer health hazards (Schmidt et al., 2016; Schmidt et al., 2018; Tournas & Niazi, 2018). In parallel, consumer`s acceptance for conventional food preservation is decreasing continuously. Consumers demand “clean-label” products combined with high standards in terms of food safety and quality (Figiel & Kufel, 2016). Therefore, research has to focus on new, natural

approaches to ensure microbial safety, in order to meet consumer`s desire and nutritional demands.

One approach of bio-preservation that recently received a lot of research interest are plant-derived antimicrobial peptides (AMPs).

One such peptide, previously reported as natural antibacterial agent is the cowpea-thionin II (Cp-thionin II). It can be found in various tissues of the plant with highest concentrations in the seeds. During germination the peptide concentration was found to decrease (Franco et al., 2006). This suggests that the peptide is part of the natural plant defense mechanism, making antifungal activity likely. To the best of the authors` knowledge, the antifungal activity of natural Cp-thionin II against *F. culmorum*, *A. niger* and *P. expansum* has not been studied previously. However, a recent study with a synthetic linear analogue of the peptide showed promising results (Thery & Arendt, 2018).

Previous studies have shown the thermal stability of selected plant defensins (Broekaert et al., 1995; Terras et al., 1992), which increases their potential as food preservatives. On the other hand, plant defensins often are sensible to the presence of cations (Vriens et al., 2014), which could be a major drawback for food applications. Regarding the mode of action of the peptide against fungal hyphae, the induction of membrane permeabilization and oxidative stress towards the fungal cells are discussed (Thery & Arendt, 2018). Another important consideration for the application as preservative is the consumer safety. Although plant defensins are usually nontoxic towards mammalian cells (Thevissen et al., 2004), depending on the amount of disulfide bonds and their hydrophobicity and amphipathicity some toxic effects have been reported (Hollmann et al., 2016; Jenssen et al., 2006). Therefore, the haemolytic activity against mammalian red blood cells is of further interest to characterise the peptide. Finally, the application of natural AMPs as food preservatives was reported by several researchers (Lucera et al., 2012; Rai et al., 2016; Rydlo et al., 2006). However, the environmental conditions and sample matrix play an essential role for the efficiency of the peptide. Hence, it is uncertain if the here investigated application as preservative during cereal storage can be successful.

The results of this study provide important information regarding the potential of AMPs in general and Cp-thionin II in particular as bio-preservative. Hence, it increases the knowledge regarding this highly promising approach to naturally reduce food losses and increase sustainability to satisfy the global nutritional requirements.

2. MATERIALS AND METHODS

2.1. Extraction and partial purification of the peptide

Extraction of the peptide from commercial organic cowpea (*Vigna unguiculata*) seeds was based on the method described by Franco et al. (2006), with some modifications. In brief, organic cowpea seeds were milled to a fine flour, using a coffee grinder, and extracted with 0.1 M HCl / 0.15 M NaCl buffer (meal : buffer ratio 1:5) under continuous stirring for 4 h at 5℃. Subsequently, the supernatant was neutralised (using NaOH), filtered (pore size 0.45 µm) and saturated with 60% ammonium sulphate. The precipitate, formed overnight, was extensively dialysed against distilled water (2.0 kDa upper cutoff, Sigma-Aldrich), lyophilised and resuspended in equilibration buffer (0.15 M Tris/HCl buffer, pH 7.0, containing 5 mM $CaCl_2$). The obtained crude extract was applied to anion exchange chromatography, using a HiTrap™ DEAE FF (1 mL) column (GE Healthcare). Chromatography was carried out using an AKTA protein purification system (GE Healthcare Life Sciences). The flow rate used was 1.0 mL/min and the eluted fractions (1.0 mL) were collected. Equilibration buffer (buffer A) was used to elute the non-retained fraction, while retained proteins were displaced from the column using buffer B (0.15 M Tris/HCl buffer + 1 M NaCl, pH 7, containing 5 mM $CaCl_2$). The non-retained fraction was applied to cation exchange chromatography, using a HiTrap™ SP HP (1 mL) column (GE Healthcare) with the same conditions. The retained protein fractions were eluted with a gradient of 0 - 100% buffer B, applied over 40 min, and collected for further analysis.

After further dialysis at room temperature against distilled water and lyophilisation, the residue was redissolved in distilled water and used as stock solution for further analysis. The Pierce™ BCA Protein Assay Kit (Thermo-Fischer Scientific) was used according to the supplier`s instructions to determine the protein concentration of the

solution. The purity of the extract was assessed by SDS gel electrophoresis, using a Tris/tricine precast gel (Bio-Rad), stained with Coomassie blue G-250. Sample preparation and electrophoresis of the native and denatured (heated for 7 min in presence of 9.5 mg dithiotreitol/mL) sample were carried out according to the supplier`s instructions (Bio-Rad). The band migrating at approximately 7 kDa was used for N-terminal sequencing by Edman degradation (5 residues), carried out by LakePharma (Belmont, USA). A BLAST analysis on UniProt protein database was used to identify the peptide based on the first five amino acids determined by Edman sequencing.

2.2. Circular Dichroism (CD)

The analysis of the secondary structure of the extracted peptide was carried out using circular dichroism (CD) spectroscopy according to the method described by Liu et al. (2008). In brief, the extract was diluted in deionized water or 20 mM sodium dodecyl sulphate (SDS) to a final protein concentration of 1 mg/mL. CD measurements were performed using a Chirascan CD Spectrometer (Applied Photophysics), at 27℃ within a wavelength range of 180-260 nm. Each solution was measured in triplicate and the solvent CD was subtracted from the sample CD.

2.3. Fungal strains

Three different species of filamentous fungi commonly found on cereal products, namely *Fusarium culmorum*, *Aspergillus niger* and *Penicillium expansum,* were investigated. The fungal strains *F. culmorum* FST 4.05, *A. niger* FST4.21 and *P. expansum* FST 4.22 originated from the culture collection of School of Food and Nutritional Sciences, University College Cork (Cork, Ireland).

2.4. Antifungal activity assay

The antifungal activity of the cowpea extract was determined by following germination and growth of fungal conidia in a microtiter plate assay, as described by Van Der Weerden et al. (2008). Briefly, fungal conidia were collected from colonies

grown for 72 h on potato dextrose agar (PDA) (Sigma Aldrich) at 25℃ and diluted to a final concentration of 10^4 spores/mL in half strength potato dextrose broth (½ PDB), using a haemocytometer. Filter sterilised extracts (20 µL) and fungal spore suspension (180 µL) were combined in the wells of a 96-well microtiter plate. Final peptide concentrations in the mixture were ranging from 500 µg/mL to 6 µg/mL. Fungal growth was followed over 96 h at 25℃ by mea surement of the optical density (OD) at 620 nm (Multiscan TM, Thermo Scientific). Addition of 20 µL of 0.1% acetic acid or sterile distilled water to the fungal spore suspensions were used as negative and positive control, respectively.

Additionally, the inhibition of fungal growth was controlled on PDA plates. Fungal spore suspension was added to warm ½ PDA to a final concentration of 10^4 spores/mL and poured into a sterile petri dish (20 mL). After solidification, 4 wells were cut into the agar and filled with 50 µL of peptide solution, containing 0 – 200 µg/mL. A well containing 50 µL of 0.1% acetic acid was prepared similarly as negative control. The extrac was allowed to diffuse into the agar and the plates subsequently incubated for 3 d at 25℃. Fungal grow th inhibition was evaluated by measuring the halo around the wells.

2.5. Determination of the minimal inhibitory concentration (MIC) and half maximal inhibitory concentration (IC_{50})

After 96 h of incubation at 25℃, the MIC was deter mined as the lowest concentration of peptide that completely inhibited fungal growth. The concentration required to inhibit the fungal development by 50% (IC_{50}) was determined by non-linear regression, using the software graph PRISM (GraphPad Software, Inc., La Jolla, CA) with the microplate reader data.

2.6. Thermal stability

In order to study the thermal stability of the extract, the peptide solution was heated at 100℃ for 15 min. After cooling to room temperat ure (30 min), the antifungal activity of the extract at MIC was determined against *F. culmorum* in a 96-well microtiter plate assay, as described in section 2.3.

186

187 2.7. Effect of cations on the antifungal activity

188 The influence of various cations on the antifungal performance of the cowpea extract
189 was investigated in an antifungal assay, performed in different salt solutions as
190 described by Terras et al. (1992). *F. culmorum* spores (10^4 spores/mL) were
191 inoculated in ½ PDB, containing 100 mM NaCl, 50 mM KCl, 5 mM $CaCl_2$ or 5 mM
192 $MgCl_2$, respectively. After addition of the peptide solution (50 µg/mL), fungal growth
193 was followed in a microtiter plate assay, as described in section 2.3.

194

195 2.8. Membrane permeabilization assay

196 A membrane permeabilization assay on *F. culmorum* hyphae to characterise the
197 mode of action of the peptide extract was carried out based on the method described
198 by Van Der Weerden et al. (2008). Briefly, fungal hyphae were grown overnight at
199 25℃ in ½ PDB from a suspension of 10^4 conidia/mL. Following centrifugation (10
200 min, 5,000 g), the hyphae were washed twice with and resuspended in synthetic
201 fungal medium (SFM; prepared as described by Rodriguez et al. (2003)). The extract
202 was added to a final peptide concentration of 100, 50 (MIC), 25 and 12.5 µg/mL,
203 respectively. Solutions of hyphae without peptide or with 1% Triton X-100 (Sigma-
204 Aldrich) were used as negative and positive controls, respectively. After incubation
205 for 2 h at 25℃, the fluorophor propidium iodide wa s added to a final concentration of
206 0.5% and the mixture was incubated for 10 min at room temperature in the dark.
207 Subsequently, fluorescence of fungal hyphae was measured using a
208 fluospectrophotometer (Varioscan® LUX reader) with excitation and emission
209 wavelengths of 488 nm and 538 nm, respectively or examined using confocal laser
210 scanning microscopy (CLSM) (Olympus) (excitation wavelength 460 -490 nm).

211

212 2.9. Induction of reactive oxygen species (ROS)

213 The measurement of ROS was carried out based on the method of Van Der
214 Weerden et al. (2008) with some modifications. *F. culmorum* hyphae (grown as
215 described above) were treated with water or cowpea extract (containing various

concentrations of peptide) for 12 h before incubation with dihydrorhodamine 123 (Sigma-Aldrich) (10 μg/mL) for 2 h. After extensive washing with 0.6 M KCl fluorescence of the hyphae was visualised using a fluorescence microscope (Olympus) (excitation wavelength 460 – 490 nm) and measured using a fluospectrophotometer with excitation and emission wavelengths of 488 nm and 538 nm, respectively. Hyphae treated with water and H_2O_2 (1% w/v) were analysed as negative and positive controls, respectively.

2.10. Haemolysis assay

The peptide solution was studied for its ability to induce haemoglobin release from fresh defibrinated sheep erythrocytes as described previously by Laverty et al. (2010). Fresh sheep red blood cells (Thermo Fischer Scientific) were washed three times with equal volumes of phosphate buffered saline, pH 7.4 (PBS). After centrifugation for 15 min at 900 g, the erythrocytes were resuspended in PBS to a final concentration of 4% (v/v). In a 96-well microtiter plate, 20 μL of peptide solution (different concentrations in PBS) and 80 μL of the erythrocyte suspension were combined and incubated for 1 h at 37℃. Subsequently, the suspension was centrifuged (10 min, 1,000 g) and the supernatant was transferred to a new microtiter plate. The release of haemoglobin was measured spectrophotometrically at 405 nm. Erythrocytes treated with 0.1% Triton X-100 in PBS and PBS alone were treated similarly as positive and negative controls, respectively. The percentage of haemolysis was calculated as published by Laverty et al. (2010).

$$\%\, Haemolysis = \frac{(Abs405\ peptide\ treatment) - (Abs405\ PBS)}{(Abs405\ 0.1\%\ triton\ X - 100) - (Abs405\ PBS)}$$

The release of haemoglobin was determined for six replicates.

2.11. Wheat grain spoilage protection

Wheat grains, supplied by Doves Farm Food Ltd. (Hungerford, UK) were disinfected according to the method described by Oliveira et al. (2012). Briefly, 300 g of grains were disinfected in 2 L 10% (w/v) hydrogen peroxide (H_2O_2) solution for 10 min with continuous stirring. Subsequently, the grains were washed for 5 min in 4 L distilled

water. This procedure was repeated once, but with only 5 min of disinfection. Immediately, the grains were moved to sterile plastic boxes and dried at room temperature for 24 h under vertical sterile laminar flow. Finally, the grains were exposed to ultraviolet light (10 min) and collected aseptically for further use.

For preparation of contaminated wheat, disinfected grains were mixed with 2% (v/w) spore suspension of *F. culmorum* (10^4 spores/mL). After 10 days of incubation at 25℃, complete fungal proliferation of the grains was visible and the grains were defined as 100% infected.

Infected and disinfected grains were mixed to samples of 100 g (dry matter), containing 5% infected kernels. Subsequently, the samples were sprayed with 2% (v/w) of sterile-filtered extract (protein concentration of 0, 25, 50 and 100 µg/mL, respectively). Glacial acetic acid was applied similarly as a control. Each sample was then divided into 6 portions and filled into sterile plastic bags. The bags were sealed, perforated with two pipette tips, containing a barrier filter to allow gas exchange, and stored at room temperature. After 0 and 6 weeks of storage, 3 portions of each sample were collected, milled to a whole grain flour (particle size < 2mm), homogenised and stored at -20℃ until further analysis.

2.12. Determination of Ergosterol

The total ergosterol content before and after storage was determined based on the method of Jedlickova et al. (2008). In brief, 10 g of milled grains were extracted with 50 mL of methanol under constant shaking at room temperature for 30 min. After centrifugation, 25 mL of the supernatant were transferred into a tube containing 3 g KOH and shaken until the KOH had fully dissolved. Subsequently, 10 mL of n-hexane were added and the mixture was incubated for 30 min at 65℃. After cooling to room temperature, 5 mL distilled water were added and the upper layer collected. The extraction with n-hexane was repeated 3 times, the combined extracts evaporated till dry and the residue re-dissolved in 5 mL of methanol before analysis by HPLC.

The RP-HPLC column used was a Nova-Pak C_{18} (300 x 3.9mm, 4µm) (Agilent Technologies). Peak-identity was verified using the UV-spectra recorded by the

DAD. The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the signal/noise (s/n) ratio. The LOD was set for s/n of 3:1 and the LOQ was set for s/n of 10:1. For calibration, ergosterol standards between 1.0 and 200 µg/mL in methanol were prepared and analysed.

2.13. Statistical analysis

Statistical analysis was carried out using Microsoft XLSTAT Version 2015.5.01. (Adinosoft Inc, New York, USA). Standard deviations were calculated for absorbance values at each peptide concentration of the extract based on triplicates, unless otherwise stated. The effect of the various salts and heat treatment on the antifungal performance of the peptide was analysed with one-way ANOVA followed by a Tukey-Kramer HSD test to identify differences relative to the control. All cases with $p<0.05$ were considered as significant. The same statistical analysis was carried out to determine individual differences in haemolysis activity for each concentration of the peptide in comparison to the negative control.

3. RESULTS AND DISCUSSION

3.1. Extraction, partial purification and identification of the cowpea peptide

The purification of the crude extract containing Cp-thionin II, obtained from the cowpea seeds, was carried out by ion-exchange chromatography using Red-Sepharose columns. The anion exchange resulted in retention of one fraction which showed no antifungal activity (data not shown). The unbound fraction was further purified by cation exchange. After disposal of the unbound fraction, 6 peaks were eluted, using 1M NaCl solution, and collected. The first 5 peaks showed no antifungal activity (data not shown) and were not investigated further. The major peak (Fig. 1, black arrow) was used for further analysis and dialysed against distilled water, lyophilised and re-dissolved to a final concentration of 10 mg/mL. This extraction method resulted in a yield of approximately 30 mg peptide per 100 g cowpea seeds. The purity of the so obtained fraction was analysed by SDS-gel electrophoresis (Fig. 1). It is visible in Figure 1 (bottom) that the denatured extract shows an intense band at approximately 6 kDa, as well as 2 bands with lower

intensity at approximately 17 and 26 kDa. In contrast, the native sample has no band migrating at 6 kDa, but therefore a very broad band at 14.5 – 16 kDa, followed by 2 bands at approximately 17 and 26 kDa with very low intensity. This shows that the main peptide of the extract, when suspended in sample buffer, occurs primarily in di- and trimers. After denaturation, the dimers were broken into the monomers visible on the gel. The 2 bands at higher molecular weight show that the purification of the cowpea peptide was only partial. However, it is also visible that the band migrating at 6 kDa has the highest intensity and hence, contains the peptide primarily responsible behaviour of the extract during subsequent analysis. In addition, the UniProt database holds no information regarding a peptide from cowpea seeds with 17 or 26 kDa that exhiits antimicrobial activity. Therefore, the results of the following sections are primarily attributed to the band migrating at 6 kDa.

The N-terminal protein sequencing of the 6 kDa peptide by Edman degradation was used to identify the extracted peptide based on the first 5 amino acid residues. The BLAST analysis (UniProt protein database) revealed that the only known peptide with the N-terminal starting sequence found here is the cowpea-thionin II. Table 1 shows a comparison of antimicrobial peptides previously extracted from cowpea seeds with the first 5 amino acid residues of the peptide characterised in this study. Based on the BLAST analysis and comparison of the sequences the extracted peptide was identified as cowpea-thionin II (Cp-thionin II).

In order to obtain further information regarding the structure of the extracted protein, circular dichroism spectroscopy was performed and the results compared to the proposed 3D model of Cp-thionin II (Figure 2). The 3D model shows the peptide as one single subunit containing 3 β-sheet and one α-helical structure. Overall, the conformation of the peptide is stabilized by 4 disulfide bonds between cysteine residues, which are displayed in ball-and-stick form. This motif (CSαβ) of Cp-thionin II is typical for native defensins (Almeida et al., 2002). In good correlation with the 3D model are the CD spectra of the extract. In both solvents (water and 20 mM SDS), a slightly positive peak was found at ~190 nm, followed by a crossover at ~200 nm and a minimum at ~210 nm. Furthermore, it is noteworthy that the positive peak is slightly bigger in water, while in SDS a much bigger negative peak was found. The shape of both graphs indicates good structured conformers, which is due to the 4 disulfide bonds stabilising the peptide. Interestingly, Thery & Arendt (2018) found similar

results in SDS but a much less structured conformation in water. The explanation behind is the lack of disulphide bonds in the synthetic linear analogue studied there. Furthermore, the peak minima indicate a propensity for helical conformations, as can be found in the 3D model. In addition, CD spectroscopy revealed a slightly higher percentage of helical conformation in SDS, combined with a slight reduction of random coils (data not shown). Overall, the differences between the conformations in the 2 solvents are marginal, as the disulphide bonds ensure a structured conformation in both solvents. In addition, the peptide solubility calculator "PepCalc" indicated good water solubility for Cp-thionin II, allowing potential use in a wide range of concentrations.

As shown by Franco et al. (2006), Cp-thionin II is a peptide consisting of 46 residues, including 8 cysteines arranged in a typical disulphide bond pattern (C1-C8 / C2-C5 / C3-C6 / C4-C7) (Lay et al., 2003). It was reported to belong to the super family of γ-thionins, also known as defensins. The molecular weight determined is with approximately 6 kDa very close to the previously reported 5.2 kDa for both natural (Franco et al., 2006) and synthetic peptide (Kraszewska et al., 2016; Thery & Arendt, 2018). Due to the impurities in the extract and the limited accuracy of the SDS-PAGE it was not possible to determine the molecular weight more accurately. Additionally, the extraction method used by Franco et al. (2006) was found to result in monomers of the defensin only. In contrast, the extraction method applied here also resulted in di- and trimers. If this has an impact on the antimicrobial performance is unclear, as the inhibiting effect of residues is not fully understood yet. Franco et al., (2006) further demonstrated the antibacterial properties of the peptide. However, to the best of the authors` knowledge and according to the PhytAMP database, no studies regarding the antifungal performance of the peptide exist. In particular, regarding the potential application as food bio-preservative, the activity against common food spoilage fungi is of interest. Also the stability against environmental stress factors and the potential health risk due to consumption were investigated in this study.

3.2. Antifungal activity of the cowpea extract

The extracted peptide Cp-thionin II showed antifungal activity against the spores of *F. culmorum* but had no significant effect on the growth of *A. niger* and *P. expansum*

(Fig. 3). Substantial fungal growth inhibition of *F. culmorum* after 96 h was achieved for peptide concentrations of 25 µg/mL or higher. Based on the results of the microtiter plate assay, the IC_{50} and MIC were determined as 40 µg/mL and 50 µg/mL, respectively (Fig. 3A). Peptide concentrations required to inhibit growth of *A. niger* and *P. expansum* were found to be much higher compared to *F. culmorum.* MIC-values against both fungi were above the highest concentration investigated here (500 µg/mL, Fig. 3B and 3C). However, spore germination and fungal growth of *A. niger* after 96 h were reduced for the highest peptide concentration tested (500 µg/mL). Growth inhibition of *P. expansum* over 96 h was found to be not significant for all concentrations tested. This demonstrates the antifungal activity of Cp-thionin II against a major food spoilage fungus, despite the relatively low activity against *A. niger* and *P. expansum*. The inhibition of fungal spore germination was further assessed on PDA plates, which confirmed the results obtained from the microtiter plate assay (data not shown).

In agreement with previous studies, the partially purified cowpea extract showed substantial antifungal activity. The peptide was previously reported by Franco et al. (2006) and Kraszewska et al. (2016) for its antibacterial activity against both Gram-negative and Gram-positive strains. Furthermore, Thery & Arendt (2018) demonstrated the antifungal activity of a synthetic, linear analogue of Cp-thionin II against fungi belonging to the genera of *Fusarium*, *Aspergillus* and *Penicillium*. An explanation for the antifungal performance of the defensin is suggested to be in the structural similarity with the human beta-defensin 3 (Kraszewska et al., 2016). However, it was further reported by Kraszewska et al. (2016) that, apart from structural similarity, the overall net charge of the peptide has a major impact on the antimicrobial performance. Additionally, other researchers reported the importance of disulfide bounds, hydrophobicity and amphipaticity for the overall antimicrobial performance (Hollmann et al., 2016, Jenssen et al., 2006).

From an evolutionary point of view, the localisation of the peptide in the seeds suggests that the peptide is originally produced to protect the germinating seed from environmental pathogens, such as the fungi studied here (Franco et al., 2006). Hence, high antimicrobial activity against the tested pathogens is plausible, making the peptide potentially a promising candidate for bio-protection.

Compared to KT43C, the linear analogue of the peptide studied by Thery & Arendt (2018), the extract containing the natural peptide showed similar antifungal activity against *P. expansum* and *A. niger*. However, the MIC against *F. culmorum* was with 50 µg/mL significantly higher than reported for the linear analogue (20 µg/mL). This discrepancy can be explained by the structural differences between synthetic and natural peptide. Firstly, as indicated by SDS electrophoresis, the natural peptide occurs not only as monomer but also as di- and trimer. As a consequence double the peptide concentration (in µg/mL) results in a similar amount of active molecules available. In addition, the peptide was only partially purified in this study and impurities in form of other peptides are visible on the SDS gel. As a result, the true concentration of Cp-thionin II would be lower than the protein concentration determined for the extract, explaining the higher concentrations required for fungal inhibition.

3.3. Effect of heat and cations on the antifungal activity of the cowpea extract

In order to gain further information regarding the properties of the cowpea extract and to estimate its potential for food applications the resistance against environmental stress factors, such as heat and ionic strength, was investigated. Therefore, the impact of these stress factors on the antifungal activity against *F. culmorum* was studied. Figure 4 shows the fungal growth curves over 96 h, incubated with cowpea extract, containing 50 µg/mL (MIC) of protein, after heat treatment (100℃, 15 min) and in presence of variou s cations (Na^+, K^+, Ca^{2+}, Mg^{2+}). No differences in spore germination inhibition were evident between the heated and unheated peptide solutions. Both treatments resulted in total inhibition of germination (10^4 conidia/mL) over 96 h in ½ PDB, while the controls (no peptide, heated and unheated) resulted in normal fungal development.

In the presence of cations, the extract lost most of its antifungal activity against *F. culmorum* when applied at its MIC (50 µg/mL). The monovalent cations Na^+ (100 mM) and K^+ (50 mM) caused a complete loss of antifungal activity. Likewise, the divalent cations Ca^{2+} or Mg^{2+} (both at 5 mM) were also found to reduce the antifungal activity of Cp-thionin II substantially.

The antifungal activity remained unaffected by heat treatment, demonstrating the thermal resistance of Cp-thionin II. The heat resistance of AMPs, including synthetic Cp-thionin II and its linear analogue was previously demonstrated by Kraszewska et al. (2016) and Thery & Arendt (2018), respectively. Different factors influencing the thermal stability of a peptide are discussed. Cp-thionin II is a small peptide with flexible order when in aqueous solution (Franco et al., 2006). This attribute contributes to a higher thermal stability, as the molecules have more freedom of movement when heated.

In agreement with the studies of Kraszewska et al. (2016) and Vriens et al. (2014), the presence of cations reduced the antifungal activity of Cp-thionin II. The increased net charge of the peptide further disturbs the hydrophilic/hydrophobic balance of the peptide, compromising its antifungal activity. As a consequence of the coverage of the peptide surface, the protein interactions are reduced, inducing a lowered antimicrobial activity. Furthermore, as a result of the ions binding to the membrane, it becomes more difficult for the peptide to find receptors on the fungal membrane to bind and exert its antifungal activity (Wu et al., 2003). Finally, the presence of cations in the medium can change the overall configuration of the peptide, causing substantial deviation from its original structure and so explaining the loss in antifungal performance (Oard & Karki, 2006).

For possible future applications, the salt sensitivity of the extract can be a major drawback, in particular for preservation of food products. However, it has to be mentioned that the concentration of NaCl (100 mM = 5.8 g/100g), the most commonly found salt in food products, was higher than in most foods. An investigation regarding the level of salt tolerated by the peptide could reveal further details regarding possible food application. Furthermore, the good thermal stability found in this study would make the extract accessible for a large field of application, including processed foods.

3.4. Mode of action

In order to identify some characteristics regarding the mode of action of Cp-thionin II in the extract, a membrane permeabilization assay was performed on *F. culmorum* hyphae. The permeabilization of the fungal cell membrane was achieved with

peptide concentrations of 12.5 µg/mL (1/4 MIC) and higher (Fig. 5). The membrane permeabilization of the peptide was found in a dose-dependent manner, hence the increased fluorescence for higher concentrations. In correlation, substantial fungal growth inhibition was only achieved for peptide concentrations of 25 µg/mL or higher. Permeabilized hyphae had significant cytoplasmic granulation at higher concentrations. However, Cp-thionin II induced permeabilization appeared to be required for inhibition but was not sufficient to cause cell death, as membrane permeabilization appeared already at concentrations which were insufficient for fungal growth inhibition. It also has to be considered, that MIC-values for the inhibition of spore germination and the killing of hyphae can vary.

Another inhibitory mechanism is the increased generation of free radicals, usually from mitochondrial source, which can lead to an excessive level of reactive oxygen species (ROS), commonly known as oxidative stress. Plant defensins have been reported to induce an overproduction of ROS as part of their antifungal performance (Vriens et al., 2014). Substantial production of ROS appeared only at the highest peptide concentration (100 µg/mL, Figure 6), which is well above the concentration required for fungal growth inhibition. This suggests that the overproduction of ROS is not a primary mechanism in the antifungal performance of Cp-thionin II against *F. culmorum*. However, at high concentrations it may be a supportive mechanism further enhancing the activity of the peptide (Hayes et al., 2013).

It is evident that the inhibitory activity of Cp-thionin II against fungal hyphae involves the permeabilization of the hyphae, leading to leakage and granulation of the plasma. Interaction of the peptide with intracellular targets, inducing oxidative stress appears only as a supportive mechanism at high concentrations. However, variations regarding the mode of action against fungi of a different class, such as *A. niger* or *P. expansum,* are likely (El-Mounadi et al., 2016). Differences in morphology and cell/wall composition could change the involvement of ROS in the antifungal performance against different fungi. The results obtained are generally in good agreement with the findings of Thery & Arendt (2018) and the values obtained for the inhibition of fungal spores.

3.6. Haemolytic activity of the cowpea extract

For possible applications as food preservative, it is essential to combine high antimicrobial activity with consumer safety. Therefore, the cytolytic activity of the extract against red blood cells of sheep was determined in order to estimate its haemolytic activity against mammalian cells. The haemolytic activity was evaluated as the release of haemoglobin after treatment with the extract in a microtiter plate assay. All concentrations investigated (up to 200 μg/mL) did not lyse the red blood cells (data not shown).

It has been shown that the presence of cholesterol is the primary reason behind the reduced sensitivity of mammalian cells towards lysis by AMPs. In contrast, fungal cell membranes contain primarily ergosterol which is more easily targeted by AMPs (Mason et al., 2007). Furthermore, it has been reported by Thevissen et al. (2004) that the cytotoxicity of plant defensins is very low. However, it needs to be mentioned, that natural AMPs are often more cytotoxic compared to their linear analogues, where the disulfide bridges are removed (Liu et al., 2008). Despite the use of a natural peptide, containing disulfide bridges, a lack of haemolytic activity was found for Cp-thionin II, which is in good agreement with the results obtained by Thery & Arendt (2018). On the other hand, it has to be considered that the assay had to be carried out in PBS buffer, which contains different salts. As shown above, the extract lost its antifungal activity in presence of cations. Hence, a possible haemolytic activity could be lost as well. Consequently, further studies regarding the safety of the extract are required before food application can be considered.

3.7. Wheat grain spoilage protection

The application of the extract as food preservative was illustrated by its use on artificially infected (*F. culmorum*) wheat grains prior to storage. The ergosterol content was measured as a marker of fungal bio-mass, to evaluate the efficiency of the peptide treatment. The untreated controls showed a substantial increase in ergosterol during the storage period (Fig. 7). The 5% infected, untreated control, in particular, had an ergosterol content below detection (<LOD) in week 0 and 42.3 ± 1.3 ppm at week 6. In contrast, the acetic acid treatment resulted in total fungal inhibition with contents < LOD before and after storage. The use of the cowpea extract reduced the fungal development at all concentrations. The use of solutions

with peptide concentrations of 25 μg/mL and 50 μg/mL resulted in 38.7 ± 1.4 ppm and 25.0 ± 0.7 ppm of ergosterol, respectively. This indicates a significantly reduced fungal bio-mass compared to the untreated control. The highest concentration tested (100 μg/mL) resulted in the total inhibition of fungal development during storage (<LOD after week 6).

The extract successfully protected the wheat grains from fungal spoilage during incubation. The use of other natural AMPs to prevent food spoilage has been previously reported to be efficient against spoilage fungi (Lucera et al., 2012; Rai et al., 2016). However the concentrations required for total inhibition of fungal development were higher than the MIC against *F. culmorum* obtained *in vitro*. This can be explained by the differences in matrices between the grains and PDB. In particular, compounds present in the outer layers of the wheat kernels could compromise the antifungal performance. Furthermore, the fungus was allowed to proliferate over the kernels before the defensin solution was applied. This can result in an increase of the concentration required for inhibition. It also has to be considered, that the MIC values for inhibition of spore germination and the inhibition of hyphae can vary significantly. However, the extract containing Cp-thionin II demonstrated great potential as a bio-preservative during cereal storage, as fungal development was completely inhibited when applied at 100 μg peptide/mL. Based on this result further application of the aqueous peptide solution appear promising and should receive more research interest in the future.

4. CONCLUSIONS

The cowpea extract containing Cp-thionin II showed antifungal activity against *F. culmorum*, a spoilage fungus commonly found on cereals and cereal products. The peptide showed typical characteristics of plant-derived defensins, including heat stability and sensitivity towards cations. Based on the characteristics of other plant defensins, high pH-stability can be assumed as well (Chan & Ng 2013). This provides a broad range of potential applications for the peptide. Furthermore, it was found to be safe towards sheep erythrocytes. These results are in good agreement with previous studies of the natural peptide (Franco et al., 2006) and synthetic linear analogue (Kraszewska et al., 2016; Thery & Arendt, 2018) of the cowpea-thionin II.

563 The application to protect fungal contaminated wheat grains during storage was
564 successful, as fungal development could be completely inhibited using a solution
565 with a peptide concentration of 100 μg/mL. This result suggests that further
566 applications of the aqueous peptide solution should be explored in future research.
567 However, despite the highly promising results of this study extensive further research
568 is required. Improvement of the extraction efficiency and purity of the extract are of
569 high importance. Also a deeper understanding of the mode of action to increase the
570 antifungal performance is required. The efficiency against other food pathogens
571 would be of major interest to fully understand the potential and limits of Cp-thionin II.
572 On the other hand, the interactions with other food constituents (e.g. proteolytic
573 enzymes) need to be considered before application as bio-preservative.
574 Furthermore, the safety of the defensin towards the consumers has to be
575 investigated for both, acute and long term toxicities.

576 In conclusion, despite the need for further extensive investigation, the extract
577 containing Cp-thionin II showed great potential for the possible application as bio-
578 preservative. As such it could be a very important tool to reduce food waste and
579 increase sustainability. Hence, it can become a valuable contribution to satisfy the
580 global nutritional requirements of future generations.

581

582 ACKNOWLEGEMENTS

583 Financial support for this research was awarded by the Irish Government under the
584 National Development Plan 2007 – 2013 through the research program
585 FIRM/RSF/CoFoRD. This research was also partly funded by the Irish Department of
586 Agriculture, Food and the Marine. Special thanks to Mr. Michael Cronin for his
587 support with the CD spectroscopy.

588

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744 FIGURE CAPTIONS

745 Figure 1: Top left: Chromatogram obtained from anion exchange column (HiTrap™
746 DEAE FF) with un-retained fraction (eluted with equilibration buffer containing 0.1 M
747 HCl and 0.15 M NaCl) transferred to cation exchange column (HiTrap™ SP HP) (top
748 right). The retained proteins were eluted with a mixture of equilibration buffer without
749 (A) and with (B) 1 M NaCl added, using a gradient from 0 – 100% B over 40 minutes.
750 The fraction used for further investigation is marked with a black arrow. Bottom: SDS
751 gel electrophoresis of the fraction obtained from cation exchange chromatography.
752 1;3 – denatured sample, 2;4 – native sample, 5 – ladder.

753 Figure 2: A) Swiss model P84920 for the predicted 3D structure of the peptide
754 cowpea-thionin II. The disulfide bonds between the cysteine residues are shown in
755 ball-and-stick form. B) Circular dichroism (CD) spectrum obtained for the cowpea
756 extract in water (broken line) and 20 mM SDS (solid line).

757 Figure 3: Fungal growth curves in presence of the cowpea extract, containing
758 various concentrations of Cp-thionin II (in μg/mL) against A) *Fusarium culmorum*
759 FST 4.05, B) *Aspergillus niger* FST 4.22, C) *Penicillium expansum* FST 4.21. All
760 values for optical density (OD) are mean values of three independent replicates ±
761 standard deviation.

762 Figure 4: Heat and cation sensitivity of antifungal activity of the cowpea extract (50
763 μg/mL). Fungal growth curves of *Fusarium culmorum* after heat treatment (15
764 minutes at 100℃) (A) and in presence of various sa lts (B – E) are mean values of
765 three independent replicates ± standard deviation.

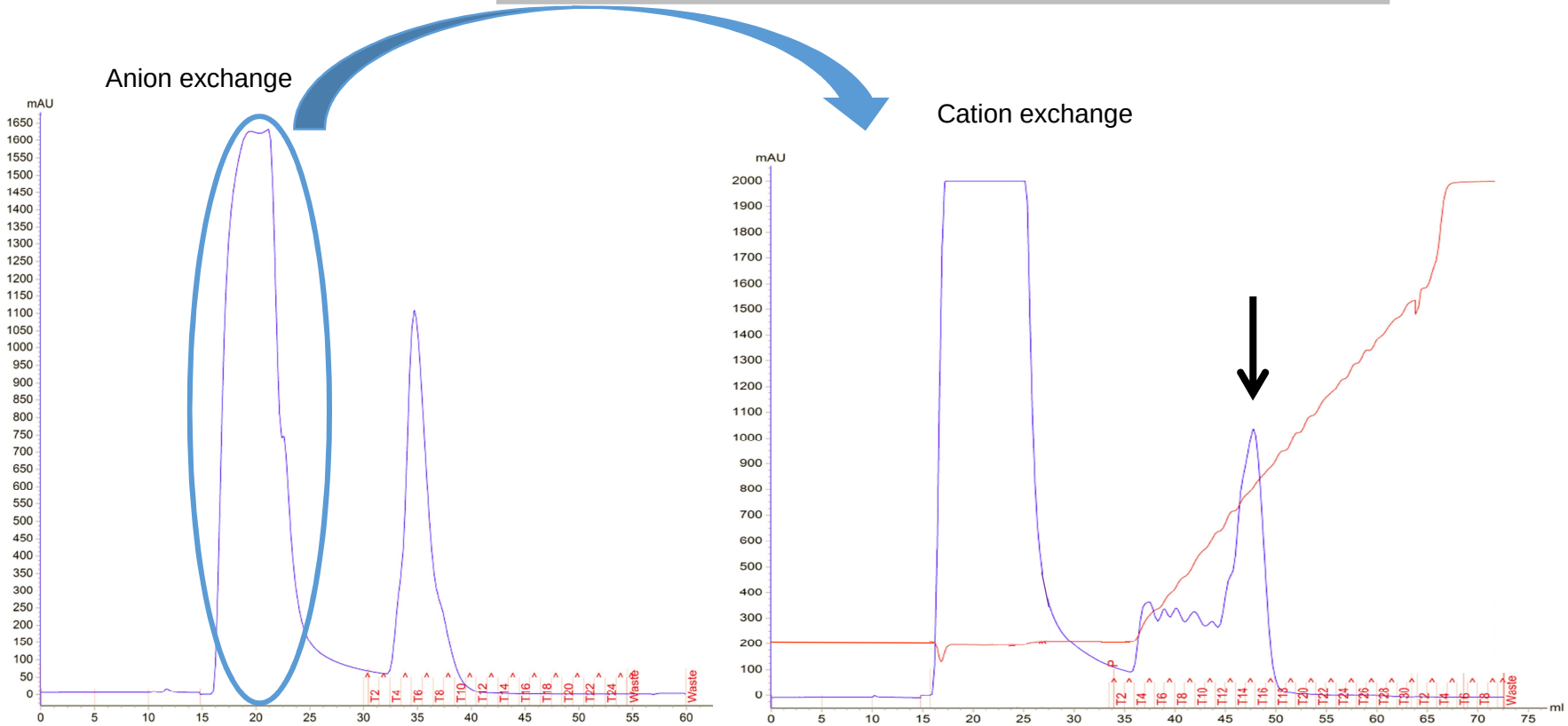
766 Figure 5: Membrane permeabilization assay. Top: After 18 hours of growth, *F.*
767 *culmorum* hyphae were treated for 2 hours with cowpea extract (0; 12.5; 25; 50 and
768 100 μg peptide/mL). Permeabilization of the fungal membrane was determined by
769 fluorescence with propidium iodid (excitation 488 nm, emission 538 nm) and
770 correlated to the percentage of inhibition. Values are the mean of three independent
771 replicates. Bottom: *F. culmorum* hyphae were observed with a confocal laser
772 scanning microscope, with an excitation wavelength 460 – 490 nm. Left: no peptide;
773 middle: 25 μg/mL; right: 100 μg/mL.

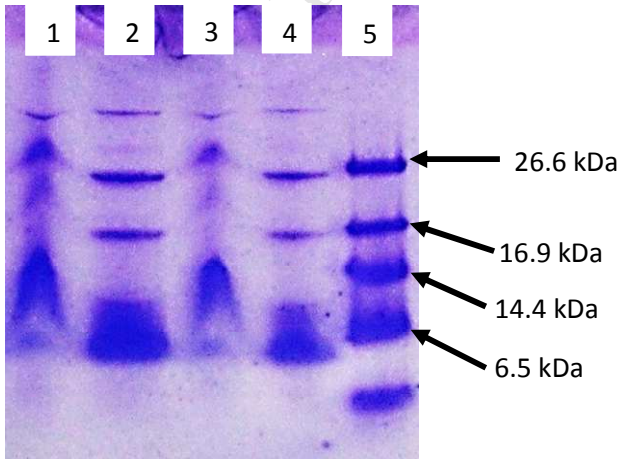
774 Figure 6: Detection of reactive oxygen species (ROS) production. Top: After 24
775 hours of growth, *F. culmorum* hyphae were treated for 12 hours with cowpea extract
776 (0; 12.5; 25; 50 and 100 μg peptide/mL). Production of ROS was determined by
777 fluorescence of DHR 123 (excitation 488 nm, emission 538 nm) and correlated to the
778 percentage of fungal growth inhibition. Each value is the mean of three independent
779 replicates. Bottom: *F. culmorum* hyphae were observed using a confocal laser
780 scanning microscope, with excitation wavelength 460 - 490 nm. Left: no peptide;
781 right: 100 μg/mL.

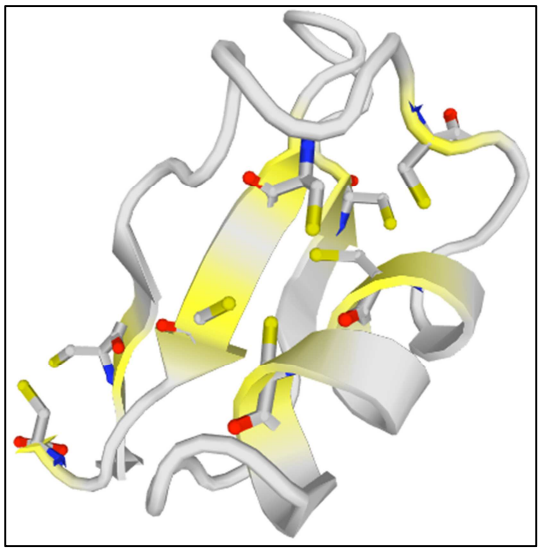
782 Figure 7: Ergosterol contents of the grain samples before (week 0, stripes) and after
783 storage (week 6, black) with standard deviation. The limit of detection (LOD) was
784 determined as 0.75 ppm and ergosterol contents determined to <LOD are shown as
785 “0 ppm” in the chart.

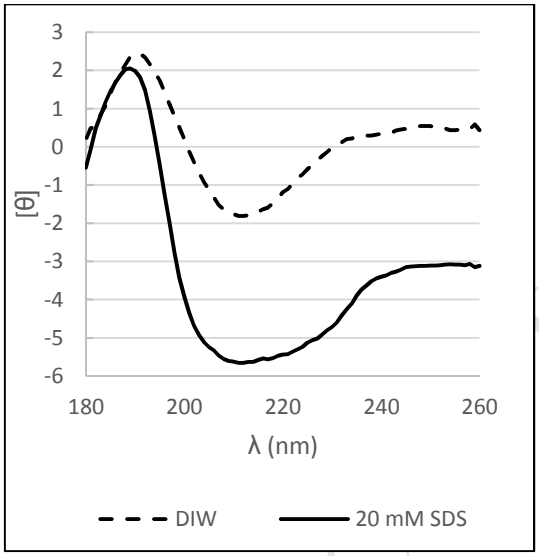
Table 1: Characteristics of the peptide extracted from cowpea seeds compared with related antimicrobial peptides (amino acids containing a disulfide bond are underlined).

Name	Source	Sequence	Activity	Function	Reference
peptide extracted here	*Vigna unguiculata*	KTCMT-	*F. cumorum* *A. niger* *P. expansum*		this study
Cp-thionin II	*Vigna unguiculata*	KTCMTKKEGWGRCLIDTTCAHSCRKYGYMG GKCQGITRRCYCLLNC	Gram-positive *S. aureus* Gram-negative *E. coli*, *P. syringae*	γ-tionin	Franco et al. (2006)
Cp-thionin	*Vigna unguiculata*	RVCESQSGFKGACTGDHNCALVCRNEGFS GGNCRGFRRRCFCTLKC	unknown	Trypsin inhibitor	Melo et al. (2002)
Linear peptide KT43C	synthetic peptide	KTCMTKKEGWGRCLIDTTCAHSCRKYGYMG GKCQGITRRCYCLLNC	*F. cumorum* *A. niger* *P. expansum*		Thery & Arendt (2018)

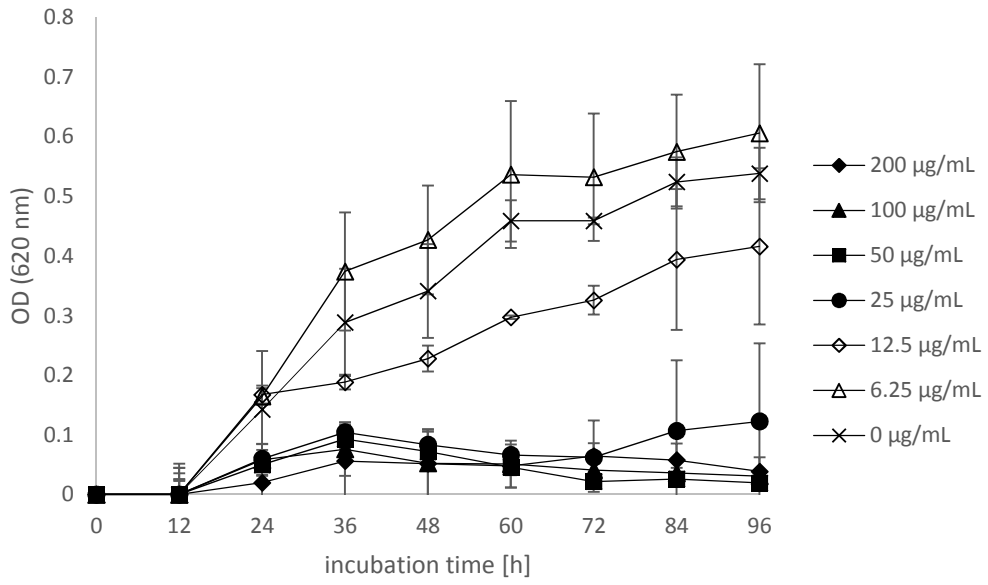




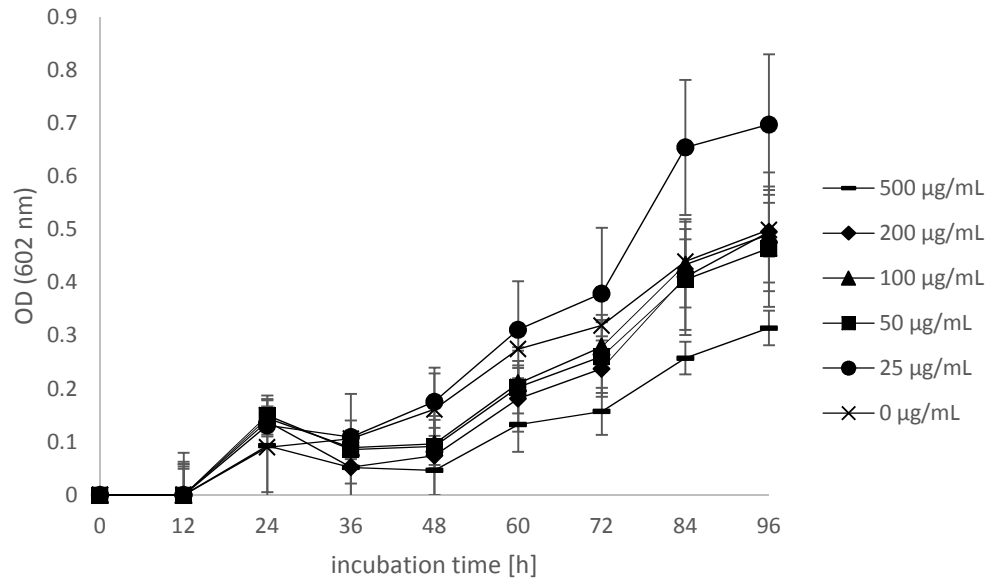




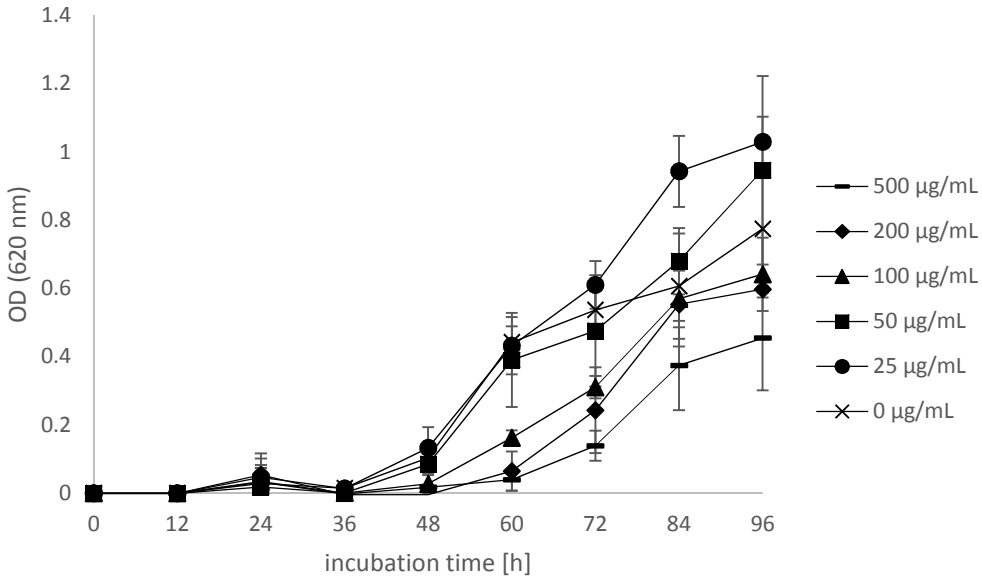
A

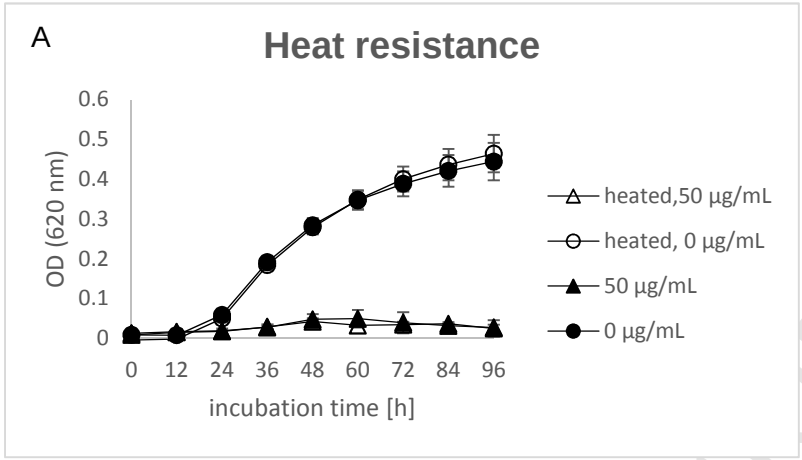

Fusarium culmorum
OD (620 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
12
24
36
48
60
72
84
96
incubation time [h]
200 µg/mL
100 µg/mL
50 µg/mL
25 µg/mL
12.5 µg/mL
6.25 µg/mL
0 µg/mL

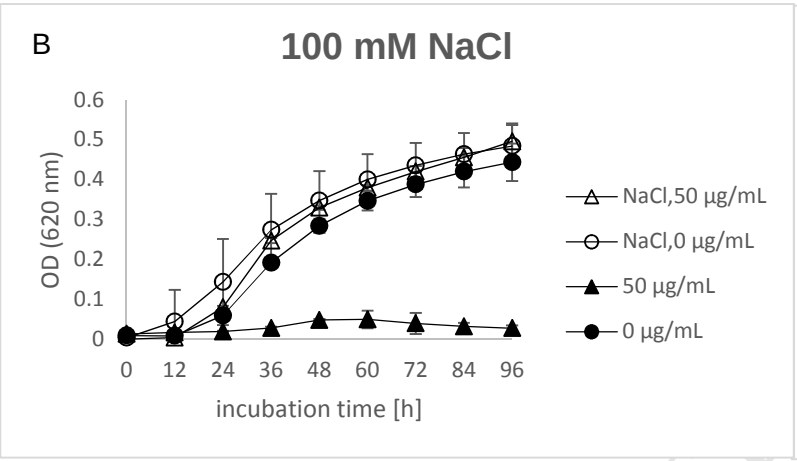
B

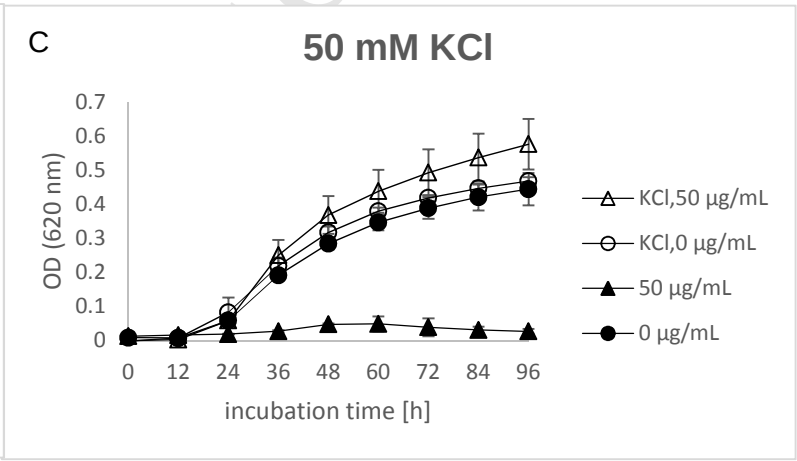

Aspergillus niger
OD (602 nm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
12
24
36
48
60
72
84
96
incubation time [h]
500 µg/mL
200 µg/mL
100 µg/mL
50 µg/mL
25 µg/mL
0 µg/mL

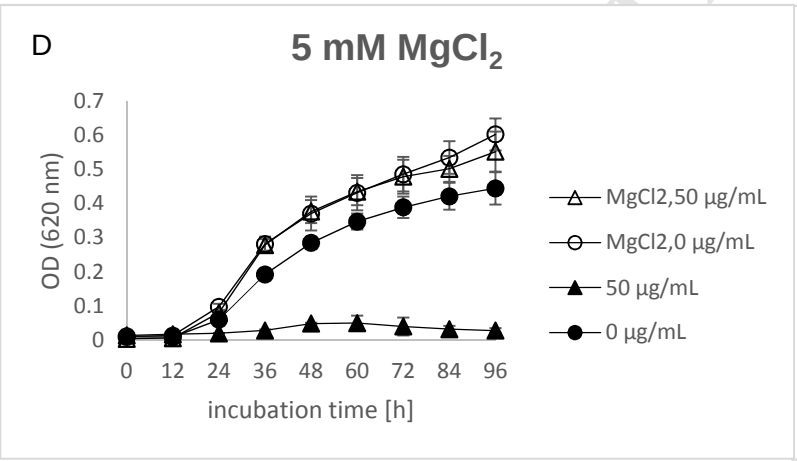
C

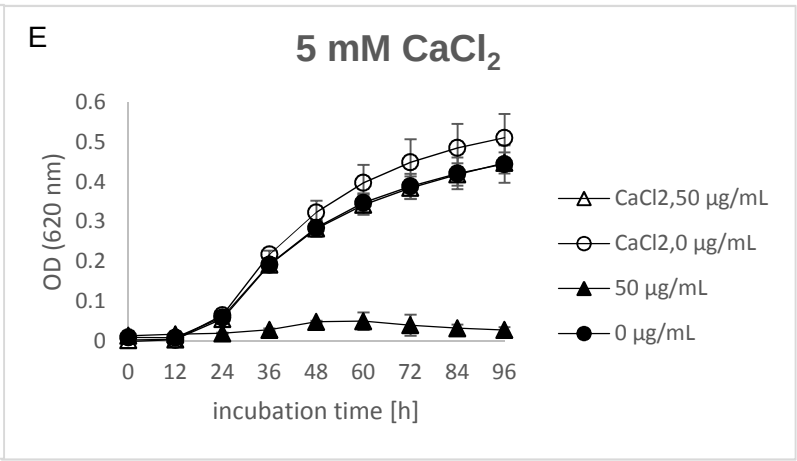

Penicillium expansum
OD (620 nm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
12
24
36
48
60
72
84
96
incubation time [h]
500 µg/mL
200 µg/mL
100 µg/mL
50 µg/mL
25 µg/mL
0 µg/mL

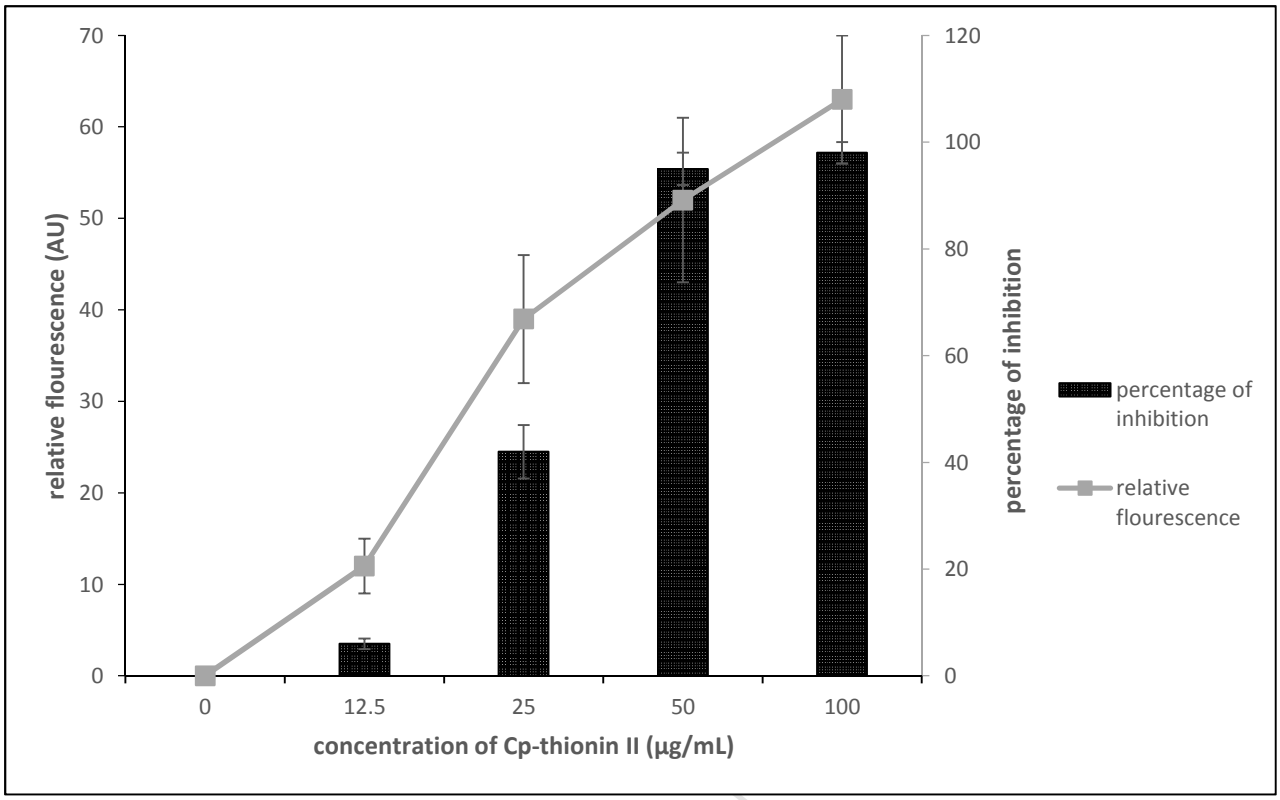

A
Heat resistance
OD (620 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0 12 24 36 48 60 72 84 96
incubation time [h]
heated,50 µg/mL
heated, 0 µg/mL
50 µg/mL
0 µg/mL

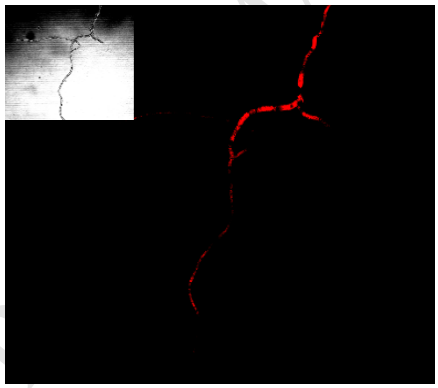

B
100 mM NaCl
OD (620 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0 12 24 36 48 60 72 84 96
incubation time [h]
NaCl,50 µg/mL
NaCl,0 µg/mL
50 µg/mL
0 µg/mL

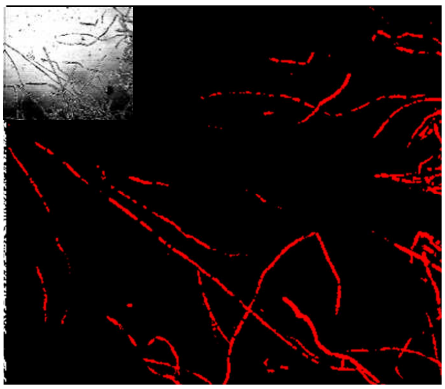

C
50 mM KCl
OD (620 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 12 24 36 48 60 72 84 96
incubation time [h]
KCl,50 µg/mL
KCl,0 µg/mL
50 µg/mL
0 µg/mL

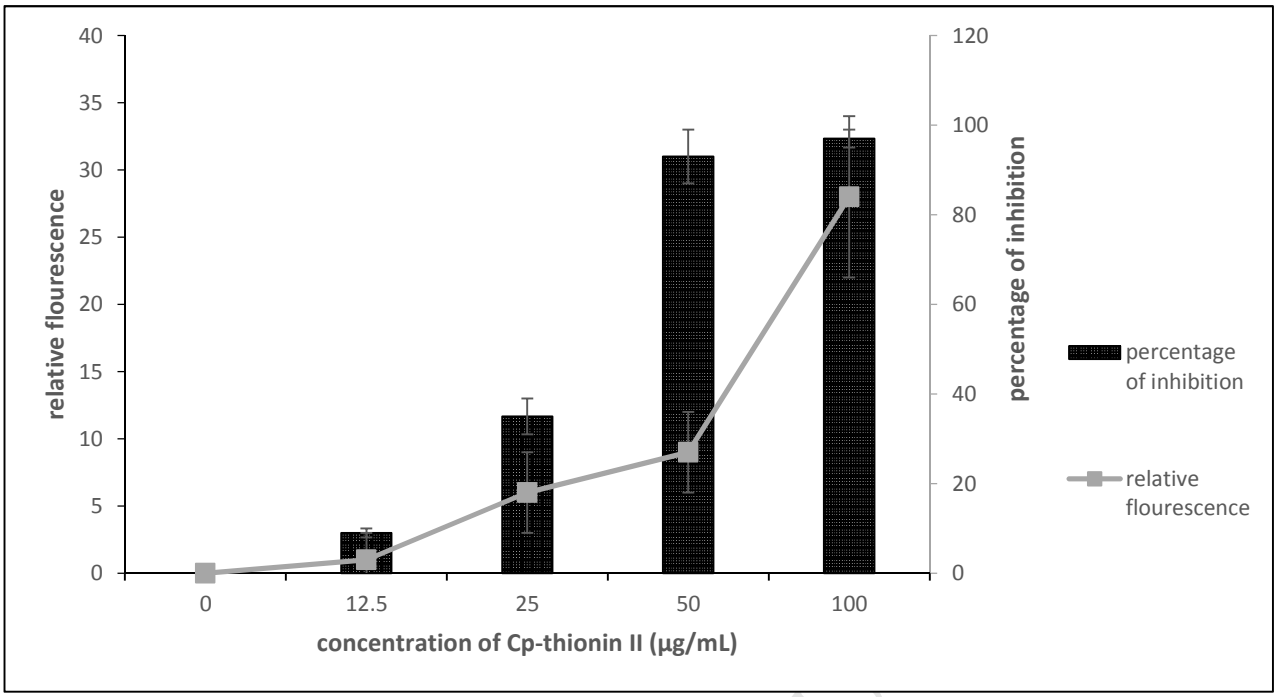

D
5 mM MgCl2
OD (620 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 12 24 36 48 60 72 84 96
incubation time [h]
MgCl2,50 µg/mL
MgCl2,0 µg/mL
50 µg/mL
0 µg/mL

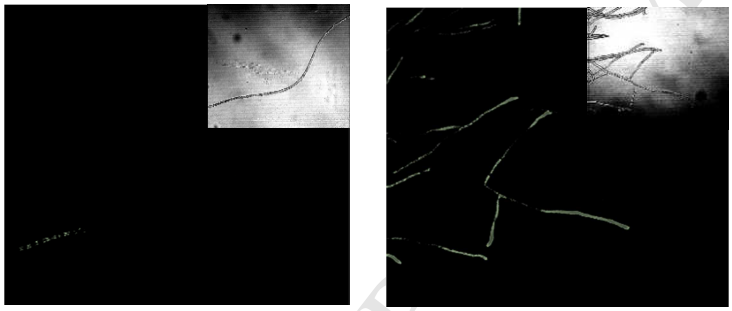

E
5 mM CaCl2
OD (620 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
0 12 24 36 48 60 72 84 96
incubation time [h]
CaCl2,50 µg/mL
CaCl2,0 µg/mL
50 µg/mL
0 µg/mL

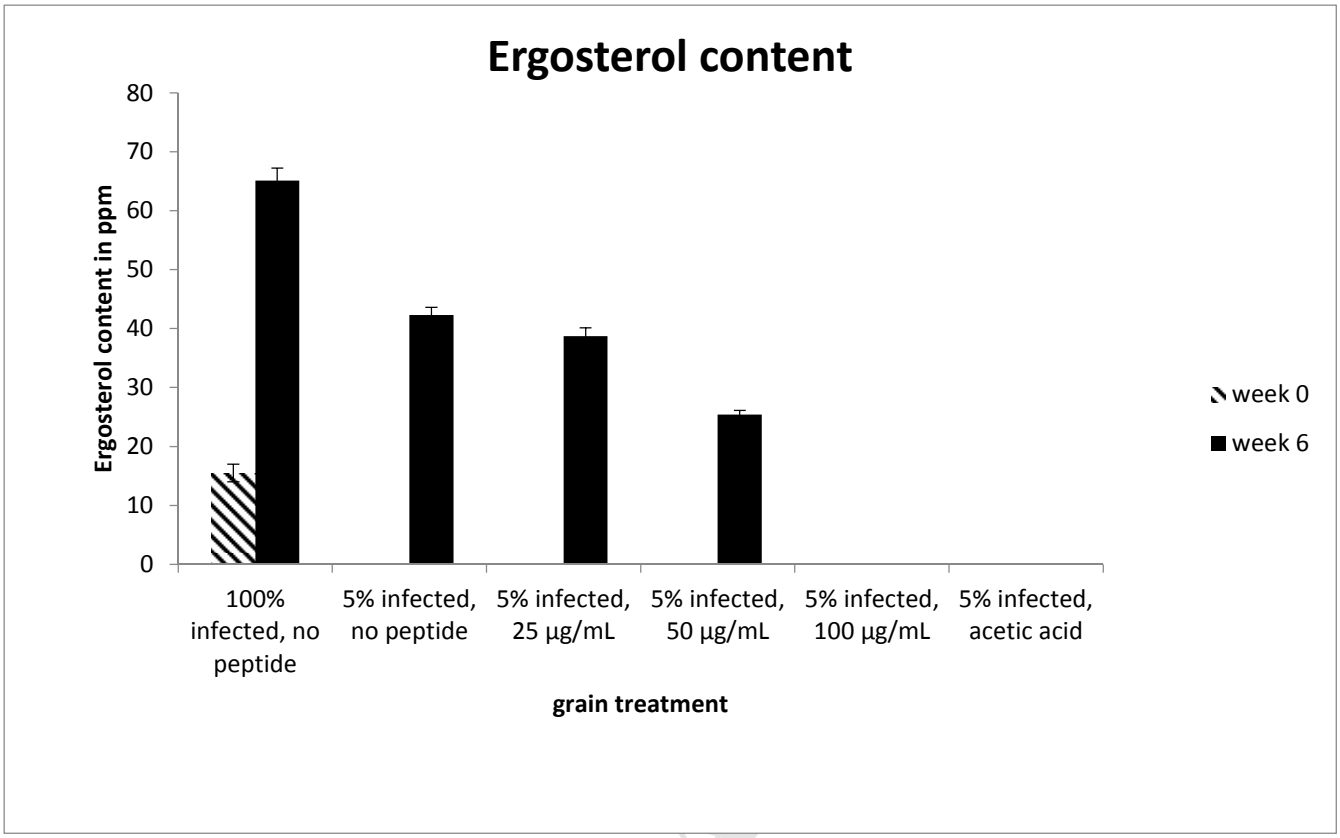












Highlights:

- Cowpea-thionin II expressed antifungal activity against *F. culmorum*
- Antifungal activity was lost due to cations but resistant against heat
- Membrane permeabilization was found to contribute to fungal inhibition
- Extract was applied to protect cereal spoilage during storage